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1 **The ABC transporter DerAB of *Lactobacillus casei* mediates resistance against insect-derived**
2 **defensins**

3

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17 **RUNNING TITLE: Defensin resistance in *Lactobacillus casei***

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21

22 **KEY WORDS:** cell envelope stress response, antimicrobial peptide resistance, ABC transporter,
23 two-component system, defensins

24 **ABSTRACT**

25 Bce-like systems mediate resistance against antimicrobial peptides in Firmicutes bacteria.
26 *Lactobacillus casei* BL23 encodes an 'orphan' ABC transporter that, based on homology to BceAB-
27 like systems, was proposed to contribute to antimicrobial peptide resistance. A mutant lacking the
28 permease subunit was tested for sensitivity against a collection of peptides derived from bacteria,
29 fungi, insects and humans. Our results show that the transporter specifically conferred resistance
30 against insect-derived cysteine-stabilized $\alpha\beta$ -defensins, and it was therefore renamed DerAB for
31 *defensin resistance* ABC transporter. Surprisingly, cells lacking DerAB showed a marked increase in
32 resistance against the lantibiotic nisin. This could be explained by significantly increased
33 expression of the antimicrobial peptide resistance determinants regulated by the Bce-like systems
34 PsdRSAB (formerly Module 09) and ApsRSAB (formerly Module 12). Bacterial two hybrid studies in
35 *E. coli* showed that DerB could interact with proteins of the sensory complex in the Psd resistance
36 system. We therefore propose that interaction of DerAB with this complex in the cell creates
37 signaling interference and reduces the cell's potential to mount an effective nisin resistance
38 response. In the absence of DerB, this negative interference is relieved, leading to the observed
39 hyper-activation of the Psd-module and thus increased resistance to nisin. Our results unravel the
40 function of a previously uncharacterized Bce-like orphan resistance transporter with pleiotropic
41 biological effects on the cell.

42 **IMPORTANCE**

43 Antimicrobial peptides (AMPs) play an important role in suppressing the growth of
44 microorganisms. They can be produced by bacteria themselves – to inhibit competitors – but are
45 also widely distributed in higher eukaryotes, including insects and mammals, where they form an
46 important component of innate immunity. In low GC Gram-positive bacteria, BceAB-like

47 transporters play a crucial role in AMP resistance but have so far been primarily associated with
48 inter-bacterial competition. Here, we show that the orphan transporter DerAB from the lactic acid
49 bacterium *Lactobacillus casei* is crucial for high-level resistance against insect-derived AMPs. It
50 therefore represents an important mechanism for inter-kingdom defense. Furthermore, our
51 results support a signaling interference from DerAB on PdsRSAB module that might prevent the
52 activation of a full nisin response. The Bce modules from *L. casei* BL23 illustrate a biological
53 paradox where the intrinsic nisin detoxification potential only arises in the absence of a defensin
54 specific ABC transporter.

55

56 INTRODUCTION

57 Antimicrobial peptides (AMPs) are found in all domains of life and constitute an important
58 aspect of the natural immune response of many organisms (1, 2). AMPs are considered as an
59 alternative to classical antibiotics, since the development of resistance has occurred to a lesser
60 extent (3, 4). One prominent mechanism of action of AMPs is binding to lipid II, leading to the
61 inhibition of bacterial cell wall biosynthesis, often followed by pore formation with the
62 concomitant release of cell metabolites and the loss of membrane potential (5-7). Most AMPs are
63 structurally very diverse, amphipathic molecules composed of 5-60 amino acids with a net positive
64 charge (<http://aps.unmc.edu/AP/main.php> (8)). Nisin and subtilin are elongated and flexible,
65 positively charged type A lantibiotics, while mersacidin is a more globular and rigid type B
66 lantibiotic with net negative charge (9). Insect AMPs such as cecropins have an α -helical structure,
67 while defensins have a cysteine-stabilized structure consisting of an N-terminal loop followed by
68 an α -helix and an antiparallel β -sheet ($CS\alpha\beta$), which are linked by disulfide bridges (10, 11).

69 Since AMPs represent common threats in microbial habitats, bacteria have developed a
70 wide range of different AMP resistance mechanisms (3, 12). General or non-specific resistance is
71 conferred by changing the bacterial surface properties. The *dlt* (D-alanyl-lipoteichoic acid) operon
72 mediates the D-alanylation of lipoteichoic and wall teichoic acids (13, 14), while expression of
73 *mprF* ("multiple peptide resistance factor") modifies anionic phospholipids of the membrane with
74 L-lysine or L-alanine (15, 16). In both cases, the resulting decrease in the net negative charge of
75 the bacterial envelope reduces the access of cationic AMPs to their surface targets, thereby
76 conferring resistance. In contrast to this general AMP resistance, ABC transporters constitute
77 compound-specific resistance determinants for AMP detoxification by actively removing the
78 peptides from their site of action (17).

79 Bce-like systems, named after BceAB from *Bacillus subtilis*, represent a unique type of
80 AMP-detoxifying ABC transporters (18, 19). They most likely consist of two nucleotide-binding
81 domain (NBDs) subunits (ATPases) and a membrane spanning domain (MSD) subunit (permease)
82 (20) and are functionally and genetically associated with BceRS-like two-component systems (TCS)
83 (21, 22). The transporter confers resistance by a target-protection mechanism, where constant
84 removal of the AMP from its cellular target keeps sufficient amounts of lipid II cycle intermediates
85 available to ensure continued cell wall synthesis (23, 24). The transporter works in a sensory
86 complex with the histidine kinase (HK), BceS (20). Signaling within this complex is activated in
87 response to transport activity (25), which is achieved by the transporter directly controlling the
88 conformational state of the HK (26). Upon activation, BceS then phosphorylates BceR, its cognate
89 response regulator (RR), which in turn induces the expression of *bceAB* and sometimes additional
90 target genes, which ensures antibiotic resistance. Since this detoxification process also removes
91 the antibiotic from its site of detection (i.e., the transport activity of the resistance transporter
92 decreases), Bce-dependent signalling is gradually switched off again (25, 26).

93 So-called ‘dual function’ BceAB-like transporters are required both for sensing the presence
94 of the AMPs (input) and their detoxification (output). However, a division of labor between Bce-
95 like transporters can be found in some systems: here, a specialized ‘sensing transporter’ perceives
96 the signal and passes this information on to its cognate TCS, which in turn leads to the expression
97 of a second ‘resistance transporter’ that removes the AMP from the cell surface (12, 27).

98 So far, the specificity of BceAB-like transporters could not be correlated with any particular
99 characteristic feature of the AMPs, such as structure, origin, modification or mechanism of action
100 (17, 21, 28). Some transporters can confer resistance against structurally very different AMPs,
101 while at the same time being able to distinguish between very similar compounds. For example,
102 the Psd system of *B. subtilis* responds to actagardine and enduracidin but not to mersacidin or

103 ramoplanin (29). Intriguingly, BceAB-like transporters can even be induced by AMPs against which
104 they do not mediate any resistance: e.g. PsdAB is induced by actagardine but does not confer
105 resistance against it (29). This behaviour indicates that sensing and removal of AMPs are two
106 separable functions of BceAB-like transporters, an assumption that has been genetically verified
107 for BceAB of *B. subtilis* (30).

108 *L. casei* BL23 encodes 17 TCSs (31), two of which, TCS09 and TCS12, are homologous to
109 BceRS from *B. subtilis*. Both systems are genomically and functionally associated with BceAB-like
110 transporters, ABC09 and ABC12 respectively, and constitute functional Bce-like units that were
111 initially referred to as Module 09 and Module 12 (32). Module 09 is a stand-alone detoxification
112 system, in which ABC09 is a dual function transporter and *abc09* is the only known target operon
113 of RR09. TCS09 responds to nisin via ABC09 and induces the expression of the transporter, which
114 then confers nisin resistance (Fig. 1). Module 09 additionally confers resistance against bacitracin,
115 plectasin and subtilin (32). Because its inducer/substrate spectrum and regulatory behaviour
116 resembles that of PsdRSAB from *B. subtilis* (29), we renamed Module 09 to PsdRSAB, for *peptide*
117 *antibiotic sensing and detoxification*. For Module 12, ABC12 is a sensing transporter that is not
118 involved in the detoxification process. In response to nisin, the cognate RR12 instead induces the
119 expression of a larger regulon that includes the *dlt* operon, the *mprF* gene and an operon encoding
120 an orphan BceAB-like ABC transporter of unknown function (Fig. 1). Module 12 deletion mutants
121 are sensitive to bacitracin, nisin, subtilin, mersacidin, plectasin and vancomycin, mainly due to the
122 impaired functionality of the Dlt system, which also renders these mutants acid sensitive (33). This
123 organisation and behaviour is similar to the Aps system of *Staphylococcus epidermidis*, which
124 regulates the expression of the *dlt* operon, the *mprF* gene and the *vraFG* ABC transporter in
125 response to AMPs (34). We therefore renamed Module 12 to ApsRSAB standing for *antimicrobial*
126 *peptide sensor*.

127 In this report, we investigated the function of the ApsR-dependent orphan ABC transporter
128 from *L. casei* BL23 in response to AMPs. A mutational study demonstrated that the orphan
129 transporter is a *defensin*-specific *resistance* transporter, which we therefore renamed DerAB, with
130 DerA (locus *LCABL_21680*) forming the NBD (ATPase) and DerB (locus *LCABL_21670*) the MSD
131 (permease) subunits of the system. Absence of a functional DerAB transporter not only increased
132 the sensitivity to defensins, but remarkably also resulted in a decreased sensitivity to nisin. This
133 surprising finding could be explained by gene expression studies in $\Delta derB$ mutant showing hyper-
134 activation of the Psd and Aps regulated systems in response to nisin. Moreover, bacterial two-
135 hybrid assays showed that DerB can interact with PsdS and PsdA. Based on these results, we
136 propose that non-productive protein-protein interactions of DerAB with the PsdRSAB sensory
137 complex might result in regulatory interference within Psd-module signal transduction that
138 modulates the induction of the nisin resistance response.

139 RESULTS

140 ***DerAB mediates resistance against insect-derived defensins but renders *L. casei* nisin sensitive***

141 ApsRSAB from *L. casei* BL23 is involved in resistance against several bacteriocins and the
142 fungal peptide plectasin, and it controls the expression of the BceAB-like orphan transporter
143 DerAB (32), which has so far not been studied with respect to its physiological role. Since all
144 known BceAB-like transporters mediate AMP resistance (17), we tested the sensitivity of a $\Delta derB$
145 strain against a wide range of different AMPs. No differences in MIC values were obtained for
146 bacitracin, mersacidin and vancomycin when comparing the $\Delta derB$ strain with the parental strain
147 BL23 (Table 1). While the absolute MIC values for plectasin ($> 40 \mu\text{g ml}^{-1}$) and subtilin (3% (v/v))
148 were also identical, the *derB* mutant showed slightly reduced growth in the presence of these
149 peptides compared to the wild type (Fig. 2A and B, respectively). Surprisingly, the $\Delta derB$ strain was

ten-fold more resistant against nisin than the parental strain (Fig. 2C). Since nisin and subtilin are structurally similar type A lantibiotics, these results were puzzling. We had previously shown that PsdAB from BL23 is involved in nisin and subtilin resistance (32), therefore we reasoned that a contribution to resistance/sensitivity mediated by DerAB should be revealed in a double mutant $\Delta derB \Delta psdB$. For subtilin, we did not observe any significant contribution of DerAB to mediating resistance (the MIC of subtilin was 0.5% (v/v) for both $\Delta psdB$ and $\Delta derB \Delta psdB$). In contrast, the MIC of nisin was 0.5 $\mu\text{g ml}^{-1}$ for BL23, 0.3 $\mu\text{g ml}^{-1}$ for the more sensitive $\Delta psdB$ and 5 $\mu\text{g ml}^{-1}$ for the highly resistant strain $\Delta derB$, while the $\Delta derB \Delta psdB$ mutant had an intermediate phenotype with an MIC of 1 $\mu\text{g ml}^{-1}$, being this double mutant more resistant to nisin than the wild type. These results demonstrate that a *derB* deletion could rescue the nisin sensitivity of a *psdB* mutant and corroborated the relevance of DerAB in the induction of a full nisin resistance response. A more detailed investigation of the nisin resistance phenotype of $\Delta derB$ strain is described below.

Since *L. casei* strains can be found in the human gastrointestinal tract (35), where it is exposed to AMPs of the innate immune defence (36), we next tested the sensitivity of the strains to human AMPs. LL37 is a human cathelicidin (37) that functions as a specific activator of the Bce-like ApeRSAB (formerly YxdJKLM) module of *B. subtilis* (38, 39), while hBD1 is a human defensin that is activated by reduction of its disulfide bridges (40, 41). We did not observe any differences in sensitivity to LL-37 or hBD1 between BL23 and $\Delta derB$ strains (Table 1). In contrast, strains carrying $\Delta apsR$, $\Delta apsB$ and $\Delta dltA$ mutations were more sensitive to LL-37 than the BL23 wild type (Fig. S1), indicating a role of ApsRSAB in resistance against LL-37, likely by regulating the activity of the Dlt system.

Lactobacilli can also be found in the microbiota of insects (42, 43). Next, we tested the sensitivity of BL23 and $\Delta derB$ strains against a collection of 18 insect-derived AMPs. Both strains were resistant against even the highest concentrations of most insect AMPs tested (Table 1).

174 However, for three defensins we could observe significant differences in resistance. The $\Delta derB$
175 mutant was slightly more sensitive to Lser-Def4 (BR090, Fig. 2D) and much more sensitive to
176 sapecin A (BR080; MIC > 160 $\mu\text{g ml}^{-1}$ for BL23, 80 $\mu\text{g ml}^{-1}$ for $\Delta derB$) and Lser-Def3 (BR092; MIC >
177 320 $\mu\text{g ml}^{-1}$ for BL23, 320 $\mu\text{g ml}^{-1}$ for $\Delta derB$) (Figs. 2E and F, respectively) than the parental strain,
178 indicating that DerAB mediates resistance against these compounds. To confirm that the
179 observed phenotype was specific to and directly caused by the loss of DerAB, we repeated the
180 sensitivity experiment for sapecin A on a strain carrying an ectopic copy of *derB* to complement
181 the $\Delta derB$ mutation. Indeed, complementation restored much of the original resistance (Fig. S2).
182 The slight remaining differences to the wild type are likely due to altered expression levels of *derB*
183 between the native and ectopic copy, as was observed for complementation of a *bceB* mutant of
184 *B. subtilis* (44). Taken together, the data indicate that DerAB specifically mediates resistance
185 against insect-derived AMPs.

186 Subsequent microscopic studies revealed significantly different morphological aberrations
187 in the $\Delta derB$ strain when challenged with different AMPs (Fig. 3 and Sup. Table 1). No
188 morphological differences were found between BL23 and $\Delta derB$ strains after exposure to BR005
189 (Fig. 3C and D), BR081, BR087, BR088, BR091 or subtilin 2% (v/v) (data not shown). In contrast, the
190 $\Delta derB$ strain was strongly affected after exposure to 400 $\mu\text{g ml}^{-1}$ BR090, showing chaining (Fig. 3J
191 and K), aberrant morphologies and cells becoming phase transparent (Fig. 3F). Although minor
192 changes were also observed in the wild type treated with BR090, these were far less pronounced
193 (Fig. 3E). After incubation with BR080 (Fig. 3G and H) or BR092 (data not shown), the chaining
194 phenotype of *derB* deletion mutant was even more pronounced (Fig. 3J and K), whilst the parental
195 strain remained unaffected. These morphological changes of the $\Delta derB$ strain after exposition to
196 BR080, BR090 and BR092 are in good agreement with the sensitivity phenotypes observed for
197 these AMPs (Fig. 2).

198 *L. casei* mutants in *apsRSAB* are defective in D-alanylation of teichoic acids, resulting in a
199 more negative surface charge and a higher AMP sensitivity of $\Delta apsR$, $\Delta apsB$ and Δdlt compared to
200 the parental strain (32). A possible explanation for the observed sensitivity of the $\Delta derB$ strain
201 could therefore be that DerAB somehow contributes to the regulation of the Dlt system through
202 ApsRS. However, measurements of the cell surface charge by cytochrome *c* binding assays (32, 45)
203 did not show any significant differences between BL23 and $\Delta derB$ strain (Fig. S3), thereby ruling
204 out this cause for the higher AMP sensitivity of the *derB* mutant.

205 Since the $\Delta derB$ strain showed the strongest sensitivity phenotypes when exposed to insect-
206 derived defensins (Fig. 2E and F), we additionally investigated the contribution of DerAB to
207 resistance against crude extracts derived from larvae of the dipteran species black soldier fly
208 *Hermetia illucens* (see Materials and Methods for their preparation), which are particularly prolific
209 sources of broad-spectrum antimicrobial substances (46). A recent transcriptome study identified
210 53 genes encoding putative AMPs from different families, half of which are putative defensins
211 (47). Amongst those, the defensin-like peptide 2 (DLP2;
212 ATCDLLSPFKVGHAACALHCIAMGRRGGWCDGRAVCNCRR) and the defensin-like peptide 4 (DLP4;
213 ATCDLLSPFKVGHAACAAHCIARGKRGGWCDKRAVCNCRK) have been characterized (48, 49). Both
214 defensins possess a CS $\alpha\beta$ structure and are active against Gram-positive bacteria. If DerAB is a
215 defensin-specific resistance transporter, its absence should render *L. casei* also sensitive to
216 *H. illucens* extracts. Indeed, the $\Delta derB$ strain was over 100-fold more sensitive than the parental
217 strain (Fig. 2G; MIC is 5 % (v/v) for BL23 and 0.04 % (v/v) for $\Delta derB$), thereby confirming its role in
218 conferring resistance against AMPs produced by *H. illucens*. Besides the strong effect on
219 resistance, we observed some morphological differences in the form of elongated and chaining
220 cells in the $\Delta derB$ strain after exposition to *Hermetia* larvae extracts (Fig. S4).

221 ***ApsRSAB regulates different layers of resistance against antibiotics produced by *H. illucens****
222 ***larvae***

223 The AMP resistance determinants of *L. casei* are controlled by the Psd and Aps modules:
224 the PsdRSAB system regulates the target operon *psdAB*, and the ApsRSAB system regulates the
225 expression of the *dlt* operon, *mprF* gene and *derAB* transporter (32) (Fig. 1). Because of the
226 potency of the *H. illucens* larval extracts and the strong difference in sensitivity between the *derB*
227 deletion mutant and the parental strain, we next aimed at analysing the hierarchy within the AMP
228 resistance network orchestrated by the Psd and Aps modules and the individual contributions of
229 their target genes to protecting *L. casei* against the combined challenge of *H. illucens* extracts.
230 Towards this goal, we performed a comprehensive mutational study of the individual components
231 of the AMP resistance network of *L. casei* (Fig. 4).

232 The MIC values for strains $\Delta psdR$ and $\Delta psdB$ were similar to that of the wild type strain
233 BL23 (5% (v/v)), indicating that the stand-alone Psd module is not involved in mediating resistance
234 against larval extracts (Fig. 4). In contrast, both mutants that rendered the Aps system
235 dysfunctional, $\Delta apsR$ and $\Delta apsB$ strains, were highly sensitive to *H. illucens* extracts, with an MIC
236 of 0.01% (v/v) (500-fold more sensitive than the wild type).

237 Next, we determined the MIC for all three Aps-dependent AMP resistance determinants
238 deleted individually. While both the $\Delta mprF$ and $\Delta dltA$ mutants showed a slight increase in
239 sensitivity compared to the wild type, the most marked effect was again observed in the $\Delta derB$
240 strain (Fig. 4). These data clearly show that the DerAB transporter, provides the primary resistance
241 layer against the AMPs present in the larval extract, while D-alanylation of teichoic acids and – to a
242 smaller extent – the L-lysinylation of phospholipids represent a secondary layer of resistance. In
243 agreement with this hierarchy, the MIC of a $\Delta derB\Delta dltA$ double mutant (0.02% (v/v)) was further
244 increased compared to the single mutants, that is, 16-fold more sensitive than $\Delta dltA$ strain and 2-

fold more sensitive than $\Delta derB$ strain. To rule out any minor contributions to resistance by the Psd system, we also tested a $\Delta derB \Delta psdB$ double mutant, but could not detect any changes relative to the $\Delta derB$ single mutant. Taken together, our results demonstrate that a clear hierarchical organization of multiple layers ensure protection of *L. casei* against insect-derived AMPs, and all resistance determinants relevant for counteracting the AMPs contained in *H. illucens* extract are under control of a single module: ApsRSAB.

Resistance determinant genes are transcriptionally over-expressed in $\Delta derB$ strain in response to nisin.

As mentioned above, the $\Delta derB$ strain unexpectedly showed a ten-fold increase in resistance against nisin (MIC 5 $\mu\text{g ml}^{-1}$; MIC for parental strain 0.5 $\mu\text{g ml}^{-1}$) (Table 1 and Fig. 2C). PsdAB is involved in nisin resistance and partially contributes to the hyper-resistance of $\Delta derB$ (MIC value for double mutant $\Delta derB \Delta psdB$ of 1 $\mu\text{g ml}^{-1}$). However, the main nisin resistance determinant of *L. casei* is the Dlt system, since the $\Delta dltA$ mutant was 12.5-fold more sensitive to nisin than the wild type (MIC for $\Delta dltA$ strain is 0.04 $\mu\text{g ml}^{-1}$) (32). We have previously shown that transcription of the *psdAB* and *dltA* genes is induced in a nisin-dependent manner in BL23, as are the genes *derAB* and *mprF* (32). We therefore hypothesized that the increased resistance of $\Delta derB$ strain might be due to a higher expression level of the nisin resistance determinants in this mutant. Thus, we tested the response of these genes following nisin exposure in the $\Delta derB$ strain background compared to the wild type, following the previously established procedure (32).

Expression of *psdR*, *apsB*, *apsA* and *apsR* was not induced by nisin in either strains (Fig. 5A), in agreement with the role of PsdRS and ApsRSAB in signaling rather than resistance (32). Strikingly, nisin exposure of the $\Delta derB$ strain led to a notable increase of induction of nisin resistance genes compared to the BL23 wild type (Fig. 5A): the genes encoding the transporters (*psdA*, *psdB* and *derA*) were up to ten-fold stronger induced in the $\Delta derB$ strain than in the wild

269 type. Likewise, *dltA* and *mprF* also responded more strongly (~ 2.5-fold and ~ 3.5-fold,
270 respectively) in the mutant. Importantly, no significant differences in gene expression were found
271 between Δ *derB* and the parental strain in reference conditions (Fig. S5), showing that the
272 increased induction was due to a hyper-response to nisin exposure and not general changes in
273 gene expression upon *derB* deletion. Moreover, since the systems involved in nisin resistance in
274 *L. casei* BL23 also mediate subtilin resistance (32), we next tested the response of these genes
275 following subtilin exposure in the Δ *derB* strain background compared to the wild type. Noticeably,
276 no significant differences in gene expression were found between Δ *derB* and the parental strain in
277 response to subtilin (Fig. 5B and Fig. S5). We therefore concluded that the increased nisin
278 resistance of Δ *derB* strain was due to an over-expression, compared to the parental strain, of the
279 nisin resistance determinants *psdAB*, *mprF* and the *dlt* operon, and that this response was specific
280 for nisin.

281 ***DerAB may interfere with signaling through spurious protein-protein interactions.***

282 We next investigated the molecular basis for the observed hyper-induction of nisin resistance
283 genes in the Δ *derB* strain. For the BceRSAB system of *B. subtilis*, it is known that signaling depends
284 on a sensory complex formed between the transport permease BceB and the histidine kinase BceS
285 (20). Moreover, signaling requires a functional transporter that is capable of ATP hydrolysis (25,
286 44), i.e. correct interaction between the permease BceB and the ATPase (BceA) subunits. Since it
287 could be possible that DerAB may somehow interfere with one or more of the nisin-responsive
288 signaling pathways in the cell, we performed bacterial two-hybrid experiments of all involved
289 protein partners to test if the permease DerB is able to interact with any of the components of the
290 Psd or Aps resistance systems (Fig. 6 and Fig. S6). In addition to the expected interaction between
291 DerB and DerA, we also obtained positive results for DerB paired with PsdA (Fig. 6). It is possible
292 that such non-cognate binding of the ATPase PsdA by DerB may reduce the ability of the PsdAB

293 transporter to trigger signaling via PsdS. Moreover, we observed clear interactions between DerB
294 and PsdS (Fig. 6). It is likely that such non-cognate interactions between the permease DerB and
295 the Psd histidine kinase would interfere with native signaling. Relief of this interference in the
296 *ΔderB* strain could then explain the observed increase in the amplitude of the nisin response and
297 resulting nisin resistance.

298 Unfortunately, similar bacterial two-hybrid experiments with the Aps proteins were less conclusive
299 because several of the fusion constructs did not even give positive results with their known
300 interaction partners (Fig. S6); consequently, these assays were not informative for potential
301 interactions between DerAB and the Aps system.

302 **DISCUSSION**

303 ABC transporters acting as detoxification mechanisms are of major importance as AMP
304 resistance systems in Firmicutes (17). In this study, we unraveled the function of DerAB in *L. casei*
305 BL23, which belongs to the BceAB-like group of transporters.

306 Our results demonstrate that from the available panel of antimicrobial compounds, four
307 CSαβ defensins (plectasin, sapecin A, LSer-Def4 and LSer-Def3) are the most relevant substrates of
308 DerAB. Such defensins are ubiquitously produced in plants, insects, mussels and fungi and form an
309 important component of the innate immunity (10). In addition to defined compounds, we also
310 demonstrated the relevance of DerAB for the survival of *L. casei* when challenged with a potent
311 mix of insect AMPs from *H. illucens* larvae. Lactobacilli grow in a variety of nutrient-rich
312 carbohydrate-containing habitats. In addition to its well-known roles in fermentation and spoilage
313 of food, *L. casei* is also found in the oral cavity, gastrointestinal and genital tracts of humans and
314 other animals (50). Moreover, different *Lactobacillus* species have been found in the microbiota of
315 insects (42, 43). It is usually assumed that lactobacilli present in food come from the food

316 processing equipment, raw foodstuffs such as plant material or faecal contamination, but it may
317 appear that insects may play a role in the dissemination of *L. casei*. A recent study has provided
318 evidence suggesting that insects are the natural reservoir of *Lactobacillus sanfranciscensis*, an
319 organism involved in sourdough fermentation (51). Interestingly, in this study, Operational
320 Taxonomic Units (OTUs) belonging to *L. casei* group were the second most numerous after
321 *L. sanfranciscensis* within lactobacilli OTUs (51). Our results suggest that possession of DerAB may
322 protect *L. casei* in different AMP-enriched environments of eukaryotic origin, including insects,
323 and thereby enable it to colonise its hosts.

324 Furthermore, by testing the sensitivity of our collection of mutants to extracts from
325 *H. illucens* larvae, which contain a very potent mixture of AMPs (46, 47), we showed that BL23
326 possesses several layers of AMP resistance. In this network, DerAB is the defensin-specific primary
327 resistance mechanism, while the Dlt system and MprF protein constitute the secondary, more
328 general resistance layer by altering the bacterial surface charge (Fig. 1). Remarkably, in
329 *Bacillus subtilis*, the response to bacitracin also consists of a hierarchy of different layers of
330 resistance: the BceAB transporter constitutes the primary (drug-sensing and highly efficient)
331 resistance determinant, and the LiaH system together with the BcrC phosphatase constitute the
332 secondary (damage-sensing and less efficient) layer of resistance (52). Thus, a clear hierarchical
333 organization of multiple layers ensure protection of both *L. casei* and *B. subtilis* against AMPs.
334 Interestingly, not only the individual components of the resistance networks are different between
335 *L. casei* (DerAB transporter, Dlt operon and MprF protein) and *B. subtilis* (BceAB transporter, LiaH
336 system and BcrC phosphatase) (52), also, the resistance layers are controlled by separate cell
337 envelope stress systems in *B. subtilis* (52), whereas all resistance determinants relevant for
338 counteracting the AMPs contained in *H. illucens* extract are under control of a single module,
339 ApsRSAB, in *L. casei*.

340 So far, the substrate specificity of BceAB-like transporters could not be related with any
341 particular feature of the respective AMPs (17, 21, 28). Our results for DerAB indicated a
342 preference for CS $\alpha\beta$ defensins, e.g. plectasin, sapecin A (BR080), LSer-Def4 (BR090) and LSer-Def3
343 (BR092). Surprisingly, DerAB does not confer resistance against the structurally closely related
344 defensins lucifensin (BR088), BR081, BR087, LSer-Def7 (BR089) and LSer-Def6 (BR091) (Table 1).
345 This indicates that DerAB can distinguish between very similar CS $\alpha\beta$ defensin substrates, a
346 situation reminiscent of the results obtained for the Psd module of *B. subtilis* (29). We performed
347 a structural analysis of all tested candidates mentioned above to further investigate this apparent
348 specificity of DerAB for some defensins (Fig. S7). The investigated defensins show a moderate
349 sequence conservation with an average sequence identity of 50 % (Fig. S8). The overall structure
350 of the defensins is well conserved with all available structures harboring a cysteine-stabilized α -
351 helix β -sheet (CS $\alpha\beta$) motif. The main structural deviations are in the N-terminal loop region, which
352 is also least well conserved, both with respect to its size and amino acid sequence (Fig. S7). No
353 obvious pattern of structural determinants distinguishing substrates from non-substrates could be
354 extracted, in line with previous studies on the substrate specificity of BceAB-like transporters (21).

355 The most puzzling result that we obtained during our initial sensitivity screen was the
356 hyper-resistance of the *derB* deletion mutant against the lantibiotic nisin. Transcriptional studies in
357 the Δ *derB* strain in response to nisin demonstrated that the absence of this transporter resulted in
358 an over-induction of *mprF*, the *dlt* operon and *psdAB*. Both the Dlt system and the PsdAB
359 transporter are crucial for nisin resistance in *L. casei* (32). Our results strongly suggest that their
360 over-expression accounts for the hyper-resistance of the Δ *derB* strain to nisin. This hypothesis is
361 supported by independent studies in other Firmicutes bacteria that demonstrated the role of *dlt*
362 expression levels for AMP resistance. Acquisition of nisin resistance in *Lactococcus lactis* IL1403
363 was partly due to higher expression levels of the *dlt* operon (53). Similarly, strains of

364 *Staphylococcus aureus* Sa113 bearing additional copies of the *dlt* operon showed an increased
365 level of D-alanine in LTA and WTA, which led to an increased nisin resistance (45).

366 In the case of the Δ *derB* strain investigated here, increased nisin resistance could, however,
367 not be explained by acquisition of additional copies of resistance genes. Instead, we could show
368 that DerB has the ability to interact with components of the Psd resistance system. We propose
369 that such spurious interactions with non-cognate protein partners likely have a negative impact on
370 signal transduction. That is, in the presence of nisin, not all PsdS histidine kinases receive a signal
371 from their cognate transporter PsdAB, because a proportion is in a non-productive complex with
372 DerB. This interference is removed upon *derB* deletion, allowing the full potential activation of
373 *psdAB* expression and thus a greater level of resistance against the AMP. Of note, whereas the
374 strain *C-derB*, carrying an ectopic copy of *derB* in the *derB* deletion mutant background,
375 complemented sensitivity to the defensin sapecin A, the restoration of nisin sensitivity was only
376 very weak (Fig. S2). Likely the ectopic copy of *derB* did not result in the same protein levels of DerB
377 in the cell and therefore the interference with normal Psd signaling was not observed. However,
378 indirect evidence supporting this interference model is available. Results of gene expression
379 previously reported (32) for mutants defective in the ApsRSAB system, and results reported here
380 for Δ *derB* (Fig. 5A and Fig. S5), showed an increase in expression of *psdAB* in the presence of nisin,
381 suggesting that a decrease in the ratio of DerAB over PsdAB results in increased PsdRSAB signal
382 transduction. Furthermore, results obtained with subtilin (Fig. 5B and Fig. S5) show that this is a
383 nisin-specific effect. To our knowledge, such a negative effect of one Bce-like resistance system on
384 the function of another has not been previously reported. Also, why this phenotype is observed
385 with nisin and not with a closely related lantibiotic such as subtilin, is still unclear.

386 We previously showed that Bce-like systems are wide-spread among Firmicutes bacteria,
387 and that many species contain multiple copies with different substrate specificities (21). Often, the

388 closest homolog to a given system is found in a different species, while the paralogs within a single
389 species can be quite distantly related (21). For the Bce-like systems characterized to date, signaling
390 between transporter and two-component system appears to be highly specific and no accidental
391 cross-talk has been reported. The example of DerAB reported here may constitute an interesting
392 evolutionary intermediate where the interaction specificity is not yet completely insulated,
393 allowing non-productive interference with the Psd system. In this scenario, in the presence of
394 nisin, the interference by DerAB should pose a significant fitness burden on the cell. However, we
395 did not observe any effects of a *derB* deletion on gene expression when cells were grown in un-
396 challenged reference conditions or after subtilin exposure (Fig. 5B and S5), suggesting that DerAB
397 has no negative effect in most situations. An alternative scenario could then be that the
398 interference by DerAB is in fact beneficial to prevent an over-reaction to nisin that could also pose
399 a fitness burden to the cell. Interestingly, a similar hidden potential in the response to nisin
400 mediated by Bce-like systems was also previously reported in *S. aureus* (54). While beyond the
401 scope of the present study, it would be interesting to investigate if, under suitable experimental
402 pressure, *L. casei* BL23 could evolve increased signaling specificity and circumvent the interference
403 from DerAB.

404 In summary, in this work we demonstrated that DerAB is a defensin-specific resistance
405 transporter that constitutes a primary layer of the *L. casei* cell envelope stress response. Its
406 expression is controlled by the Aps module that also regulates the secondary, more general, layers
407 of resistance, the Dlt system and MprF. Given the high degree of protection against insect-derived
408 AMPs, it appears likely that the physiological role of DerAB is in host-microbe interactions and may
409 allow *L. casei* to avoid innate immune defenses to colonise its eukaryotic hosts.

410 **EXPERIMENTAL PROCEDURES:**

411 ***Bacterial strains, plasmids and grown conditions***

412 Table 2 and 3 list the strains and plasmids, respectively, used in this study. *Escherichia coli*
413 DH10 β and *Lactococcus lactis* MG1363 were used as intermediate hosts for cloning purposes.
414 *E. coli* strains were grown in LB medium at 37°C with aeration. *L. lactis* strains were grown in M17
415 medium supplemented with 0.5 % (w/v) glucose at 30°C under static conditions. *L. casei* strains
416 were grown in MRS broth (Oxoid) at 37°C under static conditions. 1.5 % (w/v) agar was added to
417 prepare the corresponding solid media. Strains were stored at -80°C in their corresponding growth
418 media containing 20 % (v/v) glycerol. Ampicillin 100 $\mu\text{g ml}^{-1}$ was added to *E. coli*, and erythromycin
419 5 $\mu\text{g ml}^{-1}$ was added to *L. lactis* and *L. casei*, when required.

420 ***Construction of mutants***

421 Oligonucleotides used in this study are listed in Supplemental Table 2. Cloning in *E. coli* was
422 performed following standard methods (55). *E. coli* strains were transformed by electroporation
423 with a Gene Pulser apparatus (Bio-Rad), as recommended by the manufacturer; *L. lactis* strains
424 were transformed by electroporation (56) and *L. casei* strains were transformed as described
425 previously (57).

426 For a complete deletion of gene LCABL_21670 (ΔderB) from *L. casei* BL23, two DNA
427 fragments of approximately 700 bp upstream and downstream of the target gene were amplified
428 from genomic DNA using primers pairs RG062-063 and RG064-065 (see Sup. Table 2). All
429 subsequent steps of mutant construction were performed as previously described (32). Strain C-
430 *derB* with the complementation of the *derB* deletion was achieved by cloning the corresponding
431 gene and potential ribosome binding site (12 pb intergenic region between *derA-derB*), with
432 primer pair RG220-221 (*SpeI* and *BglIII* restriction sites; Sup. Table 2), under the constitutive P1
433 promoter in the expression vector pT1NX (58). The resulting plasmid *pT1-RBS_{derB}derB* (Table 3) was
434 used to transform *L. lactis* MG1363, and transformants were checked by sequencing of the

435 inserted fragment. Subsequently, plasmid *pT1-RBS_{derB}derB* was used to transform *L. casei* Δ *derB*,
436 resulting in strain *L. casei* *C-derB* (Table 2) which was maintained with erythromycin selection.

437 Double mutant strains Δ *derB* Δ *dltA* and Δ *derB* Δ *psdB* were obtained by insertional
438 inactivation of genes LCABL_08550 (*dltA*) and LCABL_16400 (*psdB*), respectively, in *L. casei* Δ *derB*
439 mutant background. A procedure similar to the previously described to obtain insertionally
440 inactivated single mutants of genes LCABL_08550 and LCABL_16400 was followed (32).

441 ***Bacterial two-hybrid assays***

442 We constructed C-terminal and N-terminal translational fusions of the T18 and T25
443 domains of the adenylate cyclase CyaA of *Bordetella pertussis* for each protein individually (40
444 constructs; Table 3), to test protein-protein interactions between DerAB and PsdRSAB module,
445 and between DerAB and ApsRSAB module. Fusions were tested in pairwise combinations in *E. coli*
446 BTH101 (59). For each co-transformation mixture, 3 times 10 μ l were spotted onto LB agar plates
447 containing 1 mM isopropyl- β -D-1-thio-galactopyranoside (IPTG) and 100 μ g ml⁻¹ 5-bromo-4-chloro-
448 3-indolyl- β -D-galactopyranoside (X-Gal) with selection for ampicillin and kanamycin resistance.
449 Plates were incubated at 30 °C for 48 h. Positive interaction results were identified by the
450 formation of blue colonies.

451 ***Source or preparation of eukaryotic AMPs and subtilin***

452 HBD-1 and LL-37 were purchased from Sigma-Aldrich™ (SRP3011 and 94261, respectively)
453 and were reconstituted according to manufacturer instructions. The insect AMPs shown in Table 1
454 were selected according to their reported structural and functional properties, synthesized and
455 purified to >95%. Cecropin 1 (from *Eristalis*) was synthesized by PepMic (Pepmic Suzhou, Jiangsu,
456 China). Cecropin A (from *Hyalophora* and *Aedes*), Stomoxyn (from *Stomoxys*) and Sarcotoxin IA
457 were synthesized by JPT (JPT Peptide Technologies GmbH, Berlin, Germany). The rest of the
458 peptides were synthesized by Covalab (Covalab S.A.S., Villeurbanne, France), based on the

sequence of the mature peptides. All peptides were lyophilized for storage and were resuspended and diluted in double-distilled water. Excluding *Aedes* and *Eristalis* cecropins, all other cecropins and *Stomoxys* stomoxyn were modified by the C-terminal amidation.

Larvae of the black soldier fly (*Hermetia illucens*) were obtained from a pilot rearing plant in Grimma, Saxony, Germany, and grown at 24°C in the laboratory. *H. illucens* larvae were induced as previously described (39, 46) by injecting a fungal spore suspension (spores of *Verticillium lecanii* and *Metarhizium anisopliae*) into the haemolymph and by feeding a bacterial mix (*E. coli*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *B. subtilis*) to simulate an infection and boost AMP production. The larvae were pricked as previously described (46). Aqueous larval extract from *H. illucens* was prepared as previously described (46) and was considered to be at a concentration of 100% (v/v) for the MIC assays.

B. subtilis ATCC 6633 was grown in medium A to induce subtilin production as in (60). Culture supernatant was collected and was considered to be at a concentration of 100% (v/v) for MIC assays.

Antibiotic sensitivity testing

MIC assays were performed in 96-well microtiter plates as previously described (32). Overnight cultures of the strains under study were prepared with antibiotic selection when required but, no antibiotic was added for the MIC assays. The 96-well plates were incubated at 37°C under static conditions in a Synergy™ NEOALPHAB multi-mode microplate reader (BioTek®, Winooski, VT, USA). Growth was monitored by changes in OD₅₉₅. For each strain, the MIC at 15 h was defined as the lowest antibiotic concentration where the final OD was at or below the starting OD. All experiments were performed at least in duplicate.

Microscopy

Stationary phase cell cultures assessed in MIC assays were photographed. Morphology of cultures grown for 24 h in MRS was taken as reference. Possible morphological changes on bacteria were checked after exposition for 24 h to BR005, BR080, BR081, BR087, BR088, BR090, BR091, BR092, subtilin and *H. illucens* larval extracts. The cells were observed in an Olympus AX70 microscope with phase contrast at 40x magnification. Pictures were taken with an Olympus U-TV1XC camera. Pictures were analysed using the tools implemented in the Olympus Cells Sens Dimensions 1.14 software and Corel Photo Paint X5.

The average cell length of the bacteria (n = 200), the average cell-chains length (n = 40 cell-chains) and the average number of cells per chain (n = 40 cell-chains) of the stationary phase cell cultures photographed were determined using the tools implemented in the ImageJ 1.52i software (61). Unpaired t-tests (two-tailed P value, 95% confidence intervals) comparing the values of BL23 and $\Delta derB$ under the reference condition indicated means significantly different ($P < 0.05$) for the cell-chains length (P value= 0.023) and the number of cells per chain (P value= 0.002). To determine whether the morphological phenotypes of the mutant strain upon exposition to each insect derived AMP assayed were significantly different from that of the wild type, pairwise two-way ANOVA analyses were run with GraphPad Prism 5 software, testing the values of both strains under the reference condition and after each AMP exposition. A significant difference was considered when the analysis estimated P values were below of 0.01.

Cytochrome c binding assay

Comparison of the whole-cell surface charge of the wild-type strain and mutant $\Delta derB$ was performed by a cytochrome c binding assay as in (32). Briefly, cells in stationary phase at 10^{10} CFU ml⁻¹ were incubated with 150 µg ml⁻¹ cytochrome c (Sigma) in 20 mM MOPS (morpholinepropanesulfonic acid), pH 7, for 10 min at room temperature. The mixture was centrifuged twice, and the absorbance of the supernatant (containing unbound cytochrome c) was

determined at 530 nm. The binding ratio was calculated by comparing the absorbance of each supernatant after incubation with the cells relative to the absorbance of the cytochrome c solution without bacterial cells.

Antimicrobial peptide information and structure predictions

The Antimicrobial Peptide Database (APD) (<http://aps.unmc.edu/AP/main.php>) (8) was used as a reference for the information listed in Table 1. For structural information the Protein Data Bank (www.rcsb.org) (62) or UniProt (<http://www.uniprot.org/>) (63) were used. The structures of Plectasin (PDB ID 1ZFU (64)), BR080 (sapecin A; PDB ID 1L4V (65)) and BR088 (lucifensin, PDB ID 2LLD (66)) were used. If no structure was available, the Swiss-Model webpage was used to generate structural predictions (<https://swissmodel.expasy.org/>) (67-70). The structures of BR081, BR087, BR089, BR090, BR091 and BR092 were modelled applying the Sapecin A structure (PDB: 1L4V) as template. Structural comparison was performed with YASARA View (www.yasara.org) program. Multiple sequence alignment of the defensins was performed with the CLC Main Workbench 7.7.3 program.

Reverse-transcription and quantitative real-time PCR (qRT-PCR)

Samples for RNA isolation were collected as previously described (32). Nisin 22.5 ng ml⁻¹ and subtilin 1 % were used for the induction assays. Isolation of total RNA from *L. casei* strains, synthesis of cDNA, primer design and qRT-PCR were carried out as described previously (71). Primers used are listed in Sup. Table 2. *lepA*, *ileS*, *pyrG* and *pcrA* were used as constitutive reference genes (71). Linearity and amplification efficiency for each primer pair were previously determined (32). The relative expression based on the expression ratio between the target genes and reference genes was calculated using the software tool REST (72). Every real-time PCR determination was performed at least six times.

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538 The authors declare no conflict of interest.

539 **AUTHORS CONTRIBUTIONS:**

540 A.R.-G., M.Z. and T.M. conceived the study and planned the experiments. A.R.-G analyzed the data
541 and wrote the manuscript under the supervision of T.M.; M.Z. and S.G. contributed to discussing
542 the results and the final manuscript. A.R.-G. carried out most of the experiments with *L. casei* and
543 designed the B2H constructs; C.A. contributed to the construction of the mutant strains and the
544 qRT-PCR experiments; Q.Z. performed the B2H cloning and experiments. C.L. performed the
545 structural analysis of defensins and contributed to writing of the manuscript. A.M. prepared
546 *H. illucens* larvae extracts. M.R and A. V. prepared insect derived AMPs. All authors read the paper
547 and commented on the final manuscript.

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739 **TABLES**740 **Table 1: Antimicrobial peptides used in this study and MIC values against *L. casei* BL23 and *ΔderB*.**

AMP		Class	AP Database ID ^a	Sequence	3D structure ^b	MIC ^c (15h)	
						BL23	<i>ΔderB</i>
Bacteriocins							
Bacitracin		cyclic peptide	n. r.	ICLEIKOrnIFHDN ^d (bond between Lys ₆ and Asn ₁₂)	cyclic	10	10
Nisin A		type A lantibiotic	AP00205	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	non-αβ	0.5	5
Mersacidin		type B lantibiotic	AP01206	CTFTLPGGGGVCTLTSECIC	non-αβ	10	10
Subtilin		type A lantibiotic	AP00206	WKSESLCTPGCVTGALQTCFLQTLTCNCKISK	unknown	3 ^c	3 ^c (¶)
Vancomycin		glycopeptide	n. r.	n.f.	branched tricyclic	1.7 ^c	1.7 ^c
Fungi AMPs							
Plectasin		defensin	AP00549	GFGCNGPWDEDDMQCHNHCKSIKGYKGGYCAKGGFVCKCY	combine-αβ	>40	>40 (¶)
Insect AMPs							
BR001	Cecropin A	cecropin	AP00139	KWKLFFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK*-NH2	α	>320	>320
BR002	Sarcotoxin IA	cecropin A2	AP00230	GWLKKIGKKIERVQGHTRDATIQGLGIAQQAANVAATAR*-NH2	α	>320	>320
BR003	Cecropin A (insect: <i>Aedes aegypti</i>)	cecropin	n.r.	GGLKKLGKKLEGAGKRVFNAAEKALPVVAGAKALRK	(α)	>320	>320
BR005	Stomoxyn	stomoxyn	AP00484	RGFRKHFNKLVKKVKHTISETAHVAKDTAVIAGSGAAVVAAT	α	>320	>320
BR044	LSerStomox2	stomoxyn	AP02513	GFRKRFNKLVKKVKHTIKETANVSKDVAIVAGSGVAVGAAMG	(α)	>320	>320
BR080	Sapecin A	defensin	AP00227	ATCDLLSGTGINHSACAAHCLLRGNRGGYCNGKAVCVCRN	combine-αβ	>160	80
BR081	(insect: <i>Aeschna cyanea</i>)	defensin	AP00182	GFGCPLDQMQRHRCQTITGRSGGYCSGPLKLTCTCYR	bridge	>320	>320
BR087	(insect: <i>Lucilia sericata</i>)	defensin	n. r.	ATCDLLSATGFSGTACAAHCLLIGHRGGYCNTKSVCVCRD	(combine-αβ)	>400	>400
BR088	Lucifensin	defensin	AP01532	ATCDLLSGTGVKHSACAAHCLLRGNRGGYCNGRAICVCRN	combine-αβ	>400	>400
BR089	LSer-Def7	defensin	AP02507	FTCNSYACKAHCILQGHKSGSCARINLCKCQR	bridge	>320	>320
BR090	LSer-Def4	defensin	AP02505	LTCNIDRSFCLAHCLLRGYKRGFCTVKKICVCRH	bridge	>400	>400 (¶)
BR091	LSer-Def6	defensin	AP02506	GTCSSFSSALCVVHCRVRGYPDGYCSRKGICTCRR	bridge	>400	>400
BR092	LSer-Def3	defensin	AP02504	ATCDLLSGTGANHSACAAHCLLRGNRGGYCNSKAVCVCRN	bridge	>320	320
BR097	Cecropin A (<i>Galleria mellonella</i>)	cecropin	AP03067	KWKIFKKIEKAGRNIRDGIIKAGPAVSVVGEEAATYKGT*-NH2	(α)	>320	>320
BR098	Cecropin B (<i>G. mellonella</i>)	cecropin	AP03068	KWKFFKKIERVQGNIRDGIIKAGPAVQVVGQAATYKKG*-NH2	(α)	>320	>320
BR099	Cecropin C (<i>G. mellonella</i>)	cecropin	AP03069	RWKVFKKIERMGQHIRDGIIKAGPAVAVVGQASTIISG*-NH2	(α)	>320	>320

BR100	Gm cecropin D-like pept	cecropin D	AP00755	ENFFKEIERAGQRIRDAIISAAPAVETLAQAQKIIKGGD*-NH2	(α)	>320	>320
BR101	Et-Cec1 (<i>Eristalis tenax</i>)	cecropin	n. r.	GFLKKIGKKLEGAVQRTRDATIQTIAVAQAAANVAATAKQG	(α)	>320	>320
Human AMPs							
LL37		cathelicidin	AP00310	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	α -helix	>27	>27
hBD-1		defensin	AP00451	DHYNCVSSGGQCLYSACPIFTKIQGTCTYRGKAKCCK	combine- $\alpha\beta$	>20	>20

741 ^a Antimicrobial Peptide Database (APD3) (8) identification number. n.r., not registered in APD3; n.f., not found. ^b3D structure as annotated in

742 APD3 based in (73): **α** , AMPs with helical structures; **combine- $\alpha\beta$** , AMPs with α -helical and β -strands in the 3D structure; **non- $\alpha\beta$** , contains neither

743 α -helical nor β -strands; **bridge**, AMPs without 3D structure determined, disulfide-linked, usually β -structure. When no structural data was

744 available, structure prediction was made using the Swiss-Model webpage (<https://swissmodel.expasy.org/>) (67) and is indicated in parenthesis.

745 ^cMIC in $\mu\text{g ml}^{-1}$ except for: subtilin (% (v/v)) and vancomycin (mg/ml). (¶) indicates that the mutant is more sensitive when assays for final cell

746 densities (see Fig. 2). ^dSequence of linear peptide precursor.

747 **TABLE 2. Bacterial strains used in this study**

Strains	Description ^a	Source or reference
<i>E. coli</i> DH10β	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80d <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>endA1</i> <i>recA1</i> <i>deoR</i> Δ(<i>ara,leu</i>)7697 <i>araD139</i> <i>galU</i> <i>galK</i> <i>nupG</i> <i>rpsL</i> λ ⁻	Stratagene
<i>E. coli</i> BTH101	F ⁻ , <i>cya-99</i> , <i>araD139</i> , <i>galE15</i> , <i>galK16</i> , <i>rpsL1</i> (<i>Str</i> ^r), <i>hsdR2</i> , <i>mcrA1</i> , <i>mcrB1</i> .	Lab collection
<i>L. lactis</i> MG1363	Plasmid-free derivative of NCDO712	(74)
<i>L. casei</i> BL23	Wild type	B. Chassy, U. Illinois
<i>L. casei</i> Δ <i>psdR</i>	BL23 Δ <i>LCABL_16430</i>	(32)
<i>L. casei</i> Δ <i>apsR</i>	BL23 Δ <i>rrp1</i> (<i>LCABL_19600</i>)	(31)
<i>L. casei</i> Δ <i>derB</i>	BL23 Δ <i>LCABL_21670</i>	This study
<i>L. casei</i> C-Δ <i>derB</i>	BL23 Δ <i>LCABL_21670</i> harbouring plasmid <i>pT1-RBS_{derB}derB</i> <i>Ery</i> ^r	This study
<i>L. casei</i> Δ <i>psdB</i>	<i>LCABL_16400</i> mutant; <i>pRV16400</i> <i>Ery</i> ^r	(32)
<i>L. casei</i> Δ <i>apsB</i>	<i>LCABL_19580</i> mutant; <i>pRV19580</i> <i>Ery</i> ^r	(32)
<i>L. casei</i> Δ <i>dltA</i>	<i>LCABL_08550</i> (<i>dltA</i>) mutant; <i>pRV08550</i> <i>Ery</i> ^r	(32)
<i>L. casei</i> Δ <i>mprF</i>	<i>LCABL_24490</i> mutant; <i>pRV24490</i> <i>Ery</i> ^r	(32)
<i>L. casei</i> Δ <i>derB</i> Δ <i>psdB</i>	BL23 Δ <i>LCABL_21670</i> ; <i>LCABL_16400</i> mutant, <i>pRV16400</i> <i>Ery</i> ^r	This study
<i>L. casei</i> Δ <i>derB</i> Δ <i>dltA</i>	BL23 Δ <i>LCABL_21670</i> ; <i>LCABL_08550</i> (<i>dltA</i>) mutant, <i>pRV08550</i> <i>Ery</i> ^r	This study

748 ^a *Amp*^r, ampicillin resistance; *Ery*^r, erythromycin resistance; *Str*^r, streptomycin resistance.

TABLE 3. Vectors and plasmids used in this study

Vector or plasmid	Description ^a	Source or reference
<i>pRV300</i>	Insertional vector for <i>Lactobacillus</i> , <i>Amp</i> ^r , <i>Ery</i> ^r	(75)
<i>pT1NX</i>	Expression vector for Gram-positive bacteria harboring the constitutive P1 promoter; <i>Ery</i> ^r	(58)
<i>pRVOrPe-del</i>	<i>pRV300</i> containing fused flanking fragments upstream and downstream of <i>LCABL_21670</i>	This study
<i>pRV08550</i>	<i>pRV300</i> containing a 679-pb internal fragment of <i>LCABL_08550</i> (<i>dltA</i>)	(32)
<i>pRV16400</i>	<i>pRV300</i> containing a 975-pb internal fragment of <i>LCABL_16400</i> (<i>psdB</i>)	(32)
<i>pT1-RBS_{derB}derB</i>	<i>pT1NX</i> with cloned <i>LCABL_21670</i> (<i>derB</i>) and its ribosome binding site (<i>derA-derB</i> 12 pb intergenic region)	This study
<i>pUT18</i>	Vector for B2H, <i>Amp</i> ^r	Euromedex, BACTH System Kit Manual
<i>pUT18C</i>	Vector for B2H, <i>Amp</i> ^r	
<i>pUT18C zip</i>	Control plasmid for B2H, <i>Amp</i> ^r	
<i>pKT25</i>	Vector for B2H, <i>Kan</i> ^r	
<i>pKNT25</i>	Vector for B2H, <i>Kan</i> ^r	
<i>pKT25 zip</i>	Control plasmid for B2H, <i>Kan</i> ^r	
<i>pUT18C-HK9</i>	<i>pUT18C</i> containing histidine kinase <i>psdS</i> <i>LCABL_16420</i>	This study
<i>pKT25-HK9</i>	<i>pKT25</i> containing histidine kinase <i>psdS</i> <i>LCABL_16420</i>	This study
<i>pU-HK9-T18</i>	<i>pUT18</i> containing histidine kinase <i>psdS</i> <i>LCABL_16420</i>	This study
<i>pKN-HK9-T25</i>	<i>pKNT25</i> containing histidine kinase <i>psdS</i> <i>LCABL_16420</i>	This study
<i>pUT18C-RR9</i>	<i>pUT18C</i> containing response regulator <i>psdR</i> <i>LCABL_16430</i>	This study
<i>pKT25-RR9</i>	<i>pKT25</i> containing response regulator <i>psdR</i> <i>LCABL_16430</i>	This study
<i>pU-RR9-T18</i>	<i>pUT18</i> containing response regulator <i>psdR</i> <i>LCABL_16430</i>	This study
<i>pKN-RR9-T25</i>	<i>pKNT25</i> containing response regulator <i>psdR</i> <i>LCABL_16430</i>	This study
<i>pUT18C-Per9</i>	<i>pUT18C</i> containing permease <i>psdB</i> <i>LCABL_16400</i>	This study
<i>pKT25-Per9</i>	<i>pKT25</i> containing permease <i>psdB</i> <i>LCABL_16400</i>	This study
<i>pU-Per9-T18</i>	<i>pUT18</i> containing permease <i>psdB</i> <i>LCABL_16400</i>	This study
<i>pKN-Per9-T25</i>	<i>pKNT25</i> containing permease <i>psdB</i> <i>LCABL_16400</i>	This study
<i>pUT18C-ATP9</i>	<i>pUT18C</i> containing ATPase <i>psdA</i> <i>LCABL_16410</i>	This study
<i>pKT25-ATP9</i>	<i>pKT25</i> containing ATPase <i>psdA</i> <i>LCABL_16410</i>	This study

<i>pU-ATP9-T18</i>	<i>pUT18</i> containing ATPase <i>psdA</i> LCABL_16410	This study
<i>pKN-ATP9-T25</i>	<i>pKNT25</i> containing ATPase <i>psdA</i> LCABL_16410	This study
<i>pUT18C-HK12</i>	<i>pUT18C</i> containing histidine kinase <i>apsS</i> LCABL_19610	This study
<i>pKT25-HK12</i>	<i>pKT25</i> containing histidine kinase <i>apsS</i> LCABL_19610	This study
<i>pU-HK12-T18</i>	<i>pUT18</i> containing histidine kinase <i>apsS</i> LCABL_19610	This study
<i>pKN-HK12-T25</i>	<i>pKNT25</i> containing histidine kinase <i>apsS</i> LCABL_19610	This study
<i>pUT18C-RR12</i>	<i>pUT18C</i> containing response regulator <i>apsS</i> LCABL_19600	This study
<i>pKT25-RR12</i>	<i>pKT25</i> containing response regulator <i>apsS</i> LCABL_19600	This study
<i>pU-RR12-T18</i>	<i>pUT18</i> containing response regulator <i>apsS</i> LCABL_19600	This study
<i>pKN-RR12-T25</i>	<i>pKNT25</i> containing response regulator <i>apsS</i> LCABL_19600	This study
<i>pUT18C-Per12</i>	<i>pUT18C</i> containing permease <i>apsB</i> LCABL_19580	This study
<i>pKT25-Per12</i>	<i>pKT25</i> containing permease <i>apsB</i> LCABL_19580	This study
<i>pU-Per12-T18</i>	<i>pUT18</i> containing permease <i>apsB</i> LCABL_19580	This study
<i>pKN-Per12-T25</i>	<i>pKNT25</i> containing permease <i>apsB</i> LCABL_19580	This study
<i>pUT18C-ATP12</i>	<i>pUT18C</i> containing ATPase <i>apsA</i> LCABL_19590	This study
<i>pKT25-ATP12</i>	<i>pKT25</i> containing ATPase <i>apsA</i> LCABL_19590	This study
<i>pU-ATP12-T18</i>	<i>pUT18</i> containing ATPase <i>apsA</i> LCABL_19590	This study
<i>pKN-ATP12-T25</i>	<i>pKNT25</i> containing ATPase <i>apsA</i> LCABL_19590	This study
<i>pUT18C-OrPe</i>	<i>pUT18C</i> containing permease <i>derB</i> LCABL_21670	This study
<i>pKT25-OrPe</i>	<i>pKT25</i> containing permease <i>derB</i> LCABL_21670	This study
<i>pU-OrPe-T18</i>	<i>pUT18</i> containing permease <i>derB</i> LCABL_21670	This study
<i>pKN-OrPe-T25</i>	<i>pKNT25</i> containing permease <i>derB</i> LCABL_21670	This study
<i>pUT18C-OrATP</i>	<i>pUT18C</i> containing ATPase <i>derB</i> LCABL_21680	This study
<i>pKT25-OrATP</i>	<i>pKT25</i> containing ATPase <i>derB</i> LCABL_21680	This study
<i>pU-OrATP-T18</i>	<i>pUT18</i> containing ATPase <i>derB</i> LCABL_21680	This study
<i>pKN-OrATP-T25</i>	<i>pKNT25</i> containing ATPase <i>derB</i> LCABL_21680	This study

751 ^a Amp^r, ampicillin resistance; Ery^r, erythromycin resistance; Kan^r, kanamycin resistance.

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755 **Supplemental Table 1. Quantification of the different sensitivity phenotypes of *L. casei* BL23 and**
756 ***ΔderB* strains after exposition to insect derived AMPs.** Stationary phase cell cultures of BL23 and
757 *ΔderB* grown for 24 hours under reference conditions or in the presence of insect derived AMPs
758 were photographed (see Figure 3 in the main text). The bacterial length, cell-chain length and
759 number of cells per chain values were measured for each strain under the reference and stress
760 conditions. Pairwise two-way ANOVA statistical analysis were applied to determine the
761 contribution of the treatment and the strain to the morphological phenotypes (see Materials and
762 methods in the main text for further details).

Condition	Phenotype	Strain		ANOVA ^d		
		BL23	<i>ΔderB</i>	Cond.	Strain	Int.
Reference conditions	Bacterial length (μm) ^{a, b}	3,22 ± 1,00	3,30 ± 0,83			
	Cell-chain length (μm) ^{a, c}	7,43 ± 2,74	9,92 ± 6,19			
	Number of cells/chain ^{a, c}	2,25 ± 0,54	3,03 ± 1,39			
BR005 320 μg/ml	Bacterial length (μm) ^{a, b}	2,80 ± 0,68	2,69 ± 0,64	< 0,0001	0,8318	0,0929
	Cell-chain length (μm) ^{a, c}	6,82 ± 2,65	7,59 ± 3,12	0,0200	0,0100	0,1711
	Number of cells/chain ^{a, c}	2,45 ± 1,01	2,9 ± 1,15	0,8362	0,0004	0,3304
BR090 400 μg/ml	Bacterial length (μm) ^{a, b}	2,85 ± 0,87	2,90 ± 1,10	< 0,0001	0,3104	0,8299
	Cell-chain length (μm) ^{a, c}	8,37 ± 4,22	21,1 ± 10,2	< 0,0001	< 0,0001	< 0,0001
	Number of cells/chain ^{a, c}	2,93 ± 1,25	7,30 ± 3,55	< 0,0001	< 0,0001	< 0,0001
BR080 80 μg/ml	Bacterial length (μm) ^{a, b}	3,07 ± 0,83	2,57 ± 0,86	< 0,0001	0,0008	< 0,0001
	Cell-chain length (μm) ^{a, c}	7,82 ± 3,13	30,6 ± 21,7	< 0,0001	< 0,0001	< 0,0001
	Number of cells/chain ^{a, c}	2,55 ± 0,90	11,4 ± 7,46	< 0,0001	< 0,0001	< 0,0001

763 ^a The values represent means and standard deviations. ^b For bacterial length determination,
764 n = 200 cells per condition; ^c for the determination of the cell-chain length and the number of cells
765 per chain, n = 40 cell-chains per condition. ^d P values from pairwise two-way ANOVA analyses
766 testing the growth of BL23 and *derB* mutant strain under the reference condition and in the
767 presence of each of the three AMPs (BR005, BR090 and BR080) characterized microscopically in
768 Figure 3. Significant differences (P value <0.01) are indicated in bold characters. Cond., condition;
769 Int., interaction.

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Primer	Gene	Sequence (5' - 3') ^a
Cloning		
RG037	LCABL_08550	AGTCAAGCTTGTTCAGATTATTCGCGCACC
RG038	LCABL_08550	GACTACTAGTCTGACACTTGATTGCCTTGC
RG047	LCABL_16400	CTATAGGGCGAATTGGGTACCGCAAGCCTTCAGTATCGCCG
RG048	LCABL_16400	CTCGAGGGGGGGCCCGGTACCTCAGCCGCGTTTTGATAGCG
RG062	LCABL_21680	TTTTCTCGAGTCAGGTTTCAGGGAAAACGAC
RG063	LCABL_21680	GTGCGACCTAAAGGATCTTTTCTAGACGACGCCCTTACTTTTG
RG064	Intergenic region LCABL_21670 and LCABL_21660	CAAAAGTAAGGGGCGTCGTCTAGAAAAGATCCTTTAGGTCGC AC
RG065	LCABL_21660	AAAAGAATTCCGCCTCAAAGACTTCATGC
RG220	LCABL_21670	AAAACTAGTTTAGGCTTTTCCGCTAAGTTCTTATTG
RG221	LCABL_21670	AAAAAGATCTGGGGCGTCGTCTATGTTAACG
Cloning checking		
pRV300.fw	pRV300 vector	GTTTTCCAGTCACGAC
pRV300.rv	pRV300 vector	CAGGAAACAGCTATGAC
pT1NX.fwd	pT1NX vector	TGGATTGGATTAGTTCTTGTTG
pT1NX.rev	pT1NX vector	CTTCTCTGTCGCTATCTGTTG
RG068	LCABL_21660	TATGAAGTCGGCTTCCCGCATG
RG069	LCABL_21680	GTGAATTCGTCGGCATCATG
RG076	LCABL_21680	AACACCCGCATTGAAAGGTG
RG077	LCABL_21660	TCAGCAAAAACGTCACTGGC
LSEI1418R	LCABL_16410	GTCAACATTACTTAAATTAATAA
qRT-PCR		
lepA-F	lepA	CACATTGATCACGGGAAGTC
lepA-R	lepA	GTAATGCCACGTTACGTTT
ileS-F	ileS	ACCATTCCGGCTAACTATGG
ileS-R	ileS	TCAGGATCTTCGGATTTTCC
pcrA-F	pcrA	CGGCCAATAATGTGATTGAG
pcrA-R	pcrA	TCATCAGTTTCGCTTTGAGC
pyrG-F	pyrG	AATTGCGCTTTTCACTGATG
pyrG-R	pyrG	CGAAATGATCGACCACAATC
RG006	LCABL_19580	GGGAACGCGCATTATTGTG
RG007	LCABL_19580	TCTCGCGCTGAACAAGATCC
RG008	LCABL_21670	TTGCCGGTATTTTGGTCGGG
RG009	LCABL_21670	ATGTCCACAATACGGCTGGC
RG019	LCABL_08550	TGGTCGAGGTTTTCTTGGGC
RG020	LCABL_08550	CCGGTGTATGGGCAACATCC
RG021	LCABL_24490	GCCGGATCAGCCAAGACTTG
RG022	LCABL_24490	TTAGCATCGGTGTAACGGCG
RG027	LCABL_19590	TAGCTTTAGGTCAACGCGG
RG028	LCABL_19590	CTTGCGCTCTCAATCGTTGC
RG029	LCABL_19600	GGCAATGAATATGGGCGCTG
RG030	LCABL_19600	TAGGTTCTGTCGAAGCAAGGC
RG031	LCABL_21680	CACCCGCATTGAAAGGTGTC
RG032	LCABL_21680	GCAAGGTCGTTTTCCCTGAAC
RG033	LCABL_16410	GGACAGGATCTGAGCAACGTC
RG034	LCABL_16410	ATTGAAGGTGTCAAGCAAGTCG

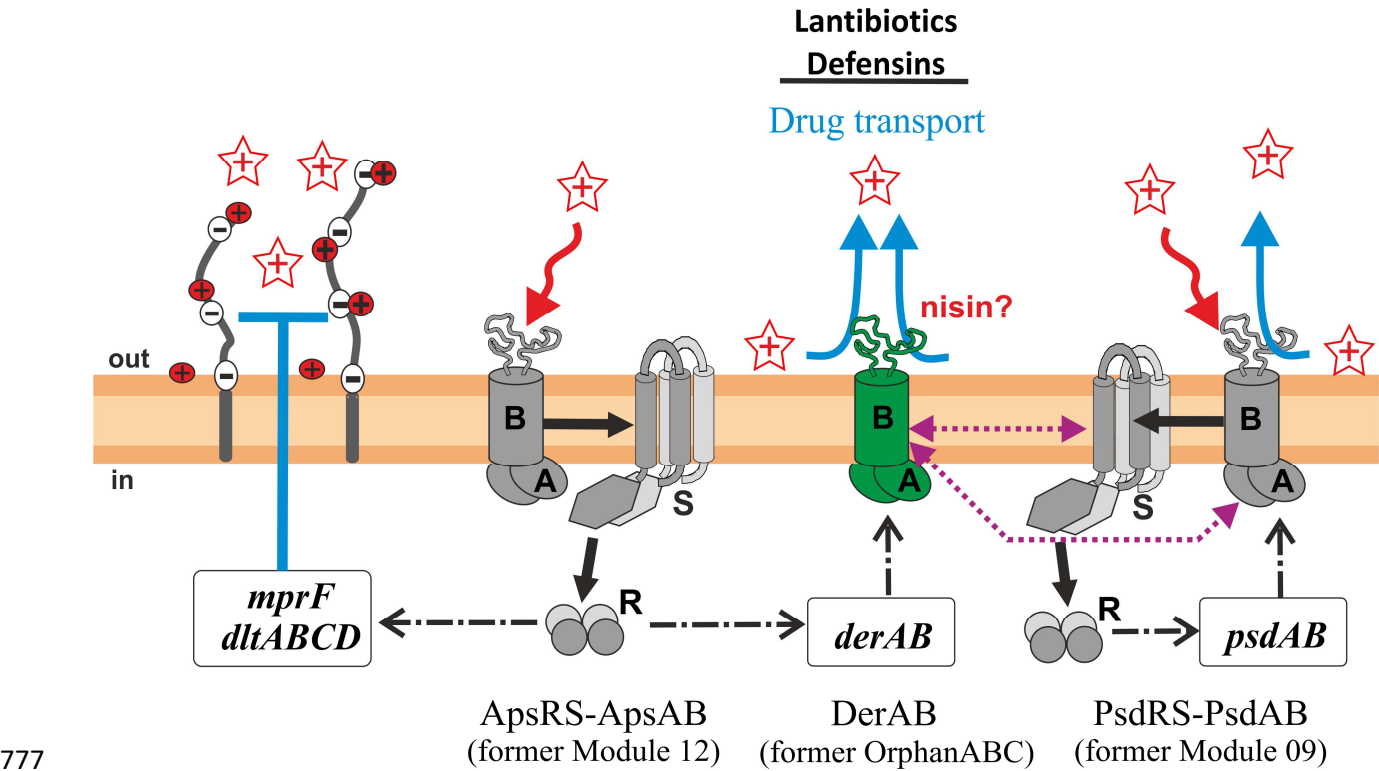
RG054	LCABL_16400	GTACCGTCCTTTCCCGCATC
RG055	LCABL_16400	CCGATGGTAATGATCCCGGC
RG056	LCABL_16430	AGCGAGTTACGCAAACACAG
RG057	LCABL_16430	CGGCTCCTAAGTTCATCGCC

B2H

TM1220	pUT18 fwd	AGCTCACTCATTAGGCACCC
TM1221	pUT18 rev	CCGTCGTAGCGGAAGTGGCG
TM1222	pUT18C fwd	TCGACGATGGGCTGGGAGCC
TM1223	pUT18C rev	AGCAGACAAGCCCGTCAGGG
TM1224	pKT25 fwd	GGCGGATATCGACATGTTCCG
TM1225	pKT25 rev	ATCGGTGCGGGCCTCTTCGC
TM1226	pKT25N fwd	GCTCACTCATTAGGCACCCC
TM1227	pKT25N rev	GGCGGAACATCAATGTGGCG
TM5702	HK9-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGATGAAAGCTTATTGCCGCTCG
TM5703	HK9-B2H-SmaI.rv1	AAAA <u>CCCGGG</u> TACTCCACTTGCCACCGCG
TM5704	HK9-B2H-SmaI.rv2	AAAA <u>CCCGGGG</u> CTCCACTTGCCACCGCGTTTG
TM5705	RR9-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGGCACAGAAAAATTTTATTGTGCGAAG
TM5706	RR9-B2H-SmaI.rv1	AAAA <u>CCCGGG</u> TCATGGCTTTGGTCCCTCAC
TM5707	RR9-B2H-SmaI.rv2	AAAA <u>CCCGGGG</u> TGGCTTTGGTCCCTCACTTGC
TM5708	ATP9-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGTCAACATTACTTAAATAAAAAATATCGA AAAAAC
TM5709	ATP9-B2H-BamHI.rv1	AAAAGGATCCTCTCATTGTCCATCGCCTGCCTTTG
TM5710	ATP9-B2H-BamHI.rv2	AAAAGGATCCTCTTGTCCATCGCCTGCCTTTG
TM5711	Per9-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGAAATTCTACTTTAAGCTCGCTGC
TM5712	Per9-B2H-SmaI.rv1	AAAA <u>CCCGGG</u> TAGCTGCGACTGGTAGCTTGG
TM5713	Per9-B2H-SmaI.rv2	AAAA <u>CCCGGGG</u> GCTGCGACTGGTAGCTTGGC
TM6396	HK12-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGCGGTTTCGTGATTATTTAAAGG
TM6397	HK12-B2H-SmaI.rv1	AAAA <u>CCCGGG</u> TCAGCTGTCTGGATGTGACCTAG
TM6398	RR12-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> GTGTTTAAATCATGATCGTAGAGG
TM6399	RR12-B2H-SmaI.rv1	AAAA <u>CCCGGG</u> GCTAAGGAACGATGTAACCTTGTC
TM6400	P12-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> GTGGAGGAGGCCATACCCGTG
TM6401	P12-B2H-SmaI.rv1	AAAA <u>CCCGGG</u> GCTAATCAATCGCCCAACGGGAAAC
TM6402	A12-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGGCAATTCTTGAAGTATCTAAGTTGAG
TM6403	A12-B2H-SmaI.rv1	AAAA <u>CCCGGG</u> GTTAATCAGATGCCACACGGG
TM6404	OrPe-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGTTAACGAACTTGCACTCGG
TM6405	OrPe-B2H-BamHI.rv1	AAAAGGATCCTTAGGCTTTTCCGCTAAGTTCTTATTG
TM6406	OrA-B2H-Xbal.fw1	AAAA <u>CTAGAG</u> ATGGACAAGCAACCTGTCGTAAC
TM6407	OrA-B2H-BamHI.rv1	AAAAGGATCCTTACTTTTGAAATGTGCCGAGTGTG
TM6408	HK12-B2H-SmaI.rv2	AAAA <u>CCCGGG</u> GCTGTCTGGATGTGACCTAGTCTG
TM6409	RR12-B2H-SmaI.rv2	AAAA <u>CCCGGG</u> GAGGAACGATGTAACCTTGTC
TM6410	P12-B2H-SmaI.rv2	AAAA <u>CCCGGG</u> GATCAATCGCCCAACGGGAAACAG
TM6411	A12-B2H-SmaI.rv2	AAAA <u>CCCGGG</u> GATCAGATGCCACACGGGTATG
TM6412	OrPe-B2H-BamHI.rv2	AAAAGGATCCTCGGCTTTTCCGCTAAGTTCTTATTG
TM6413	OrA-B2H-BamHI.rv2	AAAAGGATCCTCCTTTTGAAATGTGCCGAGTGTG

773 ^a Restriction sites are underlined. Sequences highlighted in boldface type are the sequences for
774 the CloneEZ PCR reaction as in reference (32)

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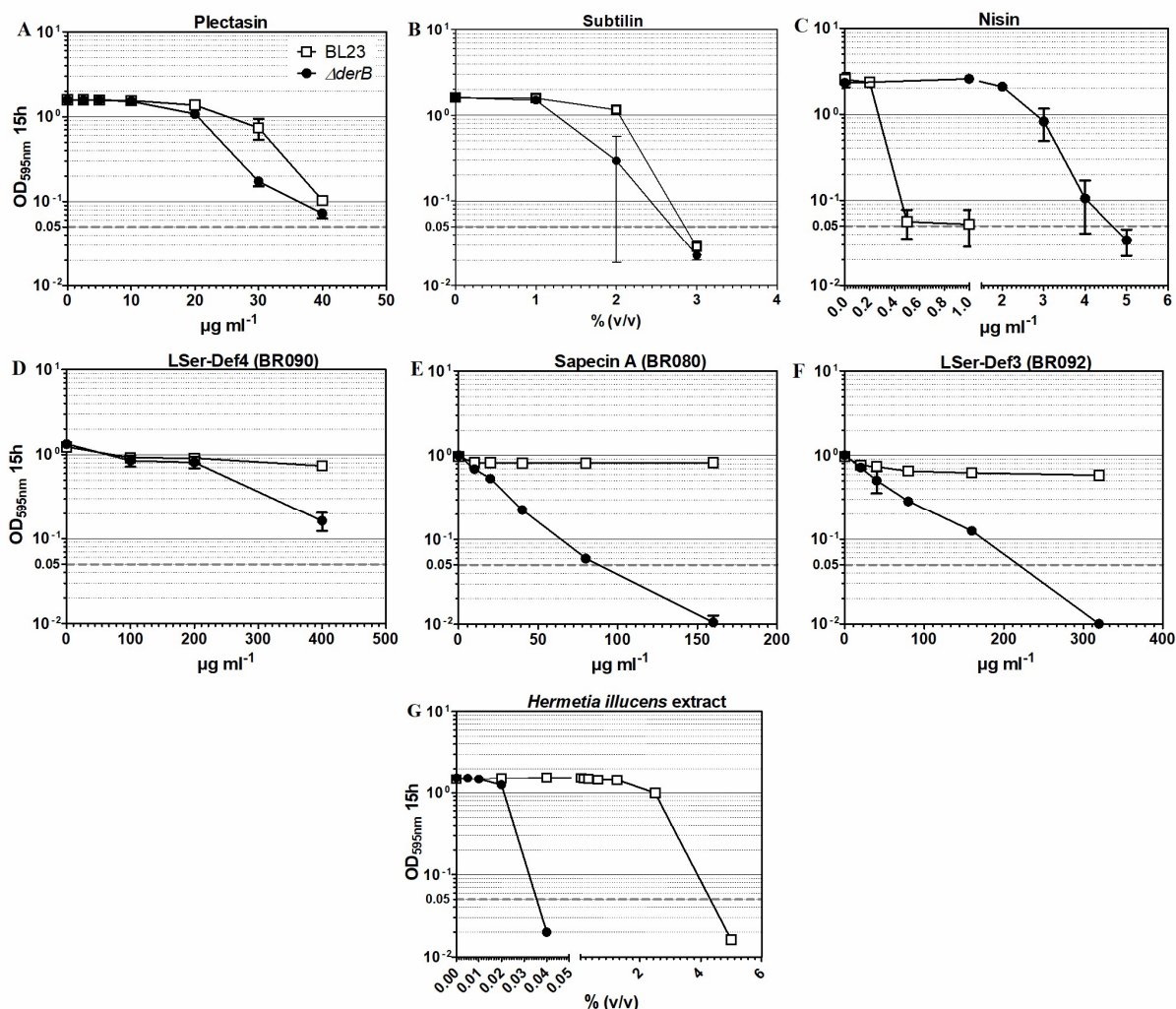


778 **Figure 1: Different layers of resistance against AMPs in *L. casei* BL23.**

779 ApsRSAB is a sensory module that regulates different layers of resistance against numerous AMPs.
780 The primary layer of AMP resistance is mediated by DerAB (drug transport) and the secondary
781 layer of resistance is mediated by the Dlt-system and MprF protein (drug repulsion). Resistance
782 mediated by DerAB is specific for defensins and – to a lesser degree - lantibiotics (see text for
783 details). PsdRSAB is a dual-function module involved in sensing and transport of various AMPs.

784 AMPs are depicted as red positively charged stars. S, histidine kinase; R, response regulator; B,
785 permease; A, ATPase. Homodimers of HKs and RRs are indicated. ABC transporters are composed
786 of a permease subunit and two ATPase domains (20). Signaling between the permeases and the
787 HKs and between the HKs and the RRs is indicated by black solid arrows. Gene activation and the
788 increased production of the transporters is indicated by black dash-dot arrows. Wave-shaped,
789 downward red arrows indicate sensing of AMPs; blue up-facing arrows indicate transport and
790 detoxification. B2H protein-protein interactions of DerB with PsdRSAB are indicated with purple
791 dotted lines. Lipoteichoic acids are indicated with grey negatively charged lines; D-alanylation of
792 teichoic acids and L-lysinylation of membrane phospholipids is indicated with positive red-filled
793 spheres; AMP charge-repulsion is indicated with a bar-headed up-facing blue line.

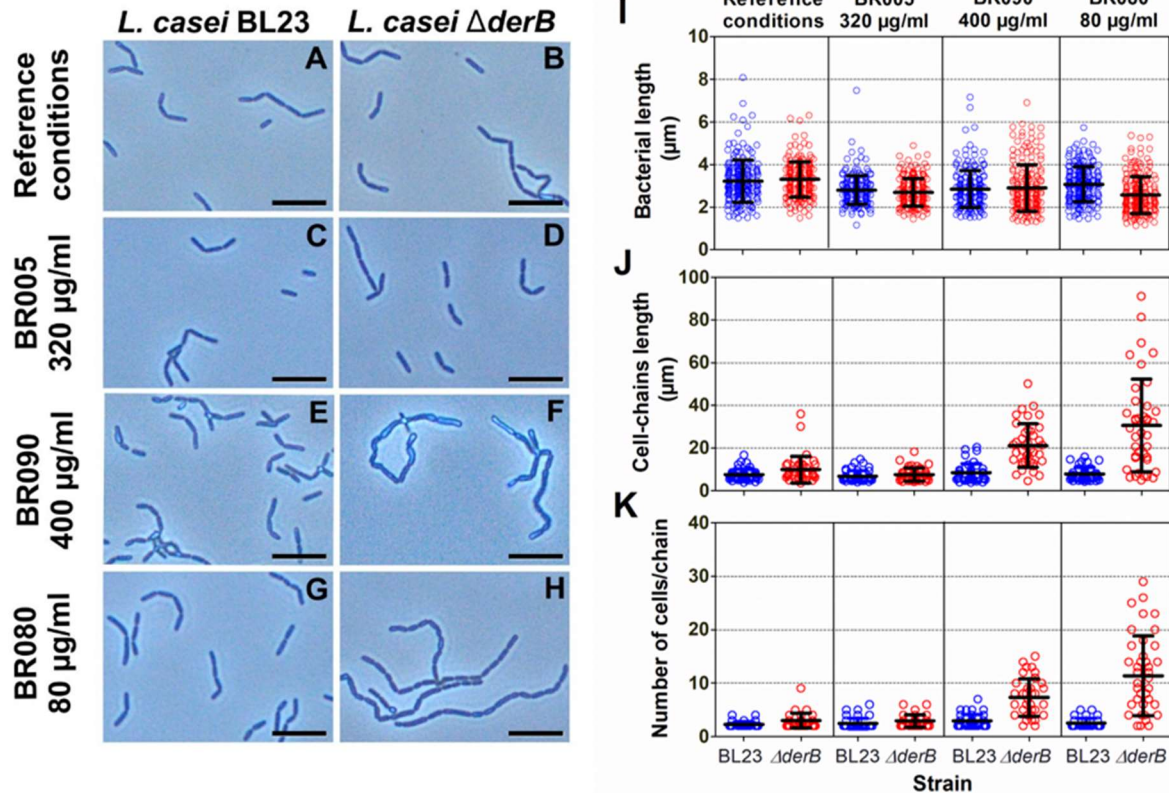
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796 **Figure 2: MIC of different AMPs for *L. casei* BL23 (white squares) and ΔderB strains (black**
 797 **circles). (A) Plectasin. (B) Subtilin. (C) Nisin. (D) LSer-Def4 (BR090). (E) Sapecin A (BR080). (F) LSer-**
 798 **Def3 (BR092). (G) Extract from *H. illucens* larvae. Strains were inoculated to an OD_{595} of 0.05**
 799 **(dashed line) in MRS with different concentrations of antibiotics. Final OD_{595} readings were taken**
 800 **after 15h incubation at 37°C (OD_{595} 15h). MIC (see Table 1) was defined at the lowest antibiotic**
 801 **concentration where the final OD was at or below the starting OD. Mean and standard deviation**
 802 **are presented of at least duplicates. A representative result of *H. illucens* extract is presented**
 803 **because due to the preparation procedure, the absolute potency of each extract (as expressed in**
 804 **%) varies significantly between individual preparations, but the relative sensitivities were very well**
 805 **reproduced in independent assays.**

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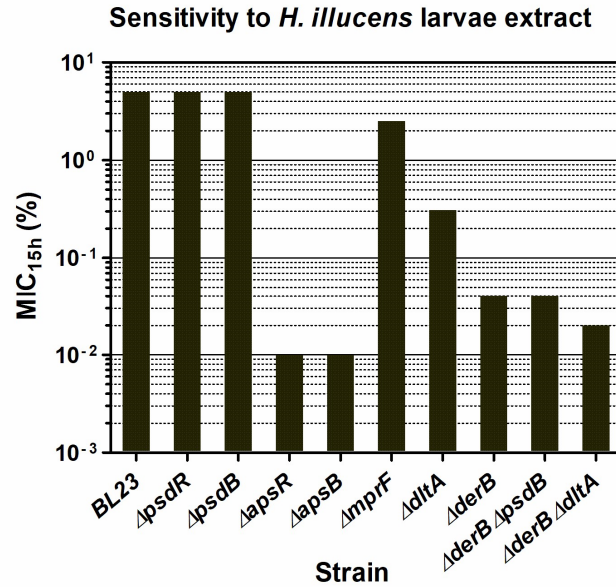


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808 **Figure 3: Sensitivity phenotypes of $\Delta derB$ strain are associated to morphological changes.**
809 **(A - H) Microscopic characterization after AMP exposition:** stationary phase cell cultures of BL23
810 (left column pictures) and $\Delta derB$ (right column pictures) grown for 24 hours under reference
811 conditions **(A and B)** or in the presence of insect derived AMPs were photographed (phase
812 contrast, 40x magnification). Selected AMPs were chosen as representative of different
813 sensitivities of the $\Delta derB$ strain relative to the wild type (see Table 1). Similar sensitivity
814 phenotype: BR005 at 320 $\mu\text{g ml}^{-1}$ **(C and D)**. $\Delta derB$ slightly more sensitive than BL23: BR090 at 400
815 $\mu\text{g ml}^{-1}$ **(E and F)**. Mutant much more sensitive than wild type: BR080 at 80 $\mu\text{g ml}^{-1}$ **(G and H)**. Scale
816 bar 10 μm .
817 **(I - K) Quantification of the morphological changes associated to the sensitivity phenotypes.**
818 **(I)**The average bacterial length (μm ; n = 200), **(J)** the average cell-chains length (μm ; n = 40 cell-
819 chains) and **(K)** the average number of cells per chain (n = 40 cell-chains) of the stationary phase
820 cell cultures photographed in **(A - H)** were determined. Means and standard deviations are
821 indicated with the horizontal and the bar-headed vertical black lines, respectively. For a detailed
822 statistical analysis of the results see Supplemental Table 1.

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826 **Figure 4: Different layers of resistance against AMPs from *H. illucens*.** MIC at 15h (expressed as
827 % (v/v) of initial extract) of *H. illucens* larvae extract against *L. casei* BL23 and derivative strains. A
828 representative dataset is shown. Due to the preparation procedure, the absolute potency of each
829 extract (as expressed in %) vary significantly between individual preparations, but the overall
830 relative sensitivities were very well reproduced in independent assays.

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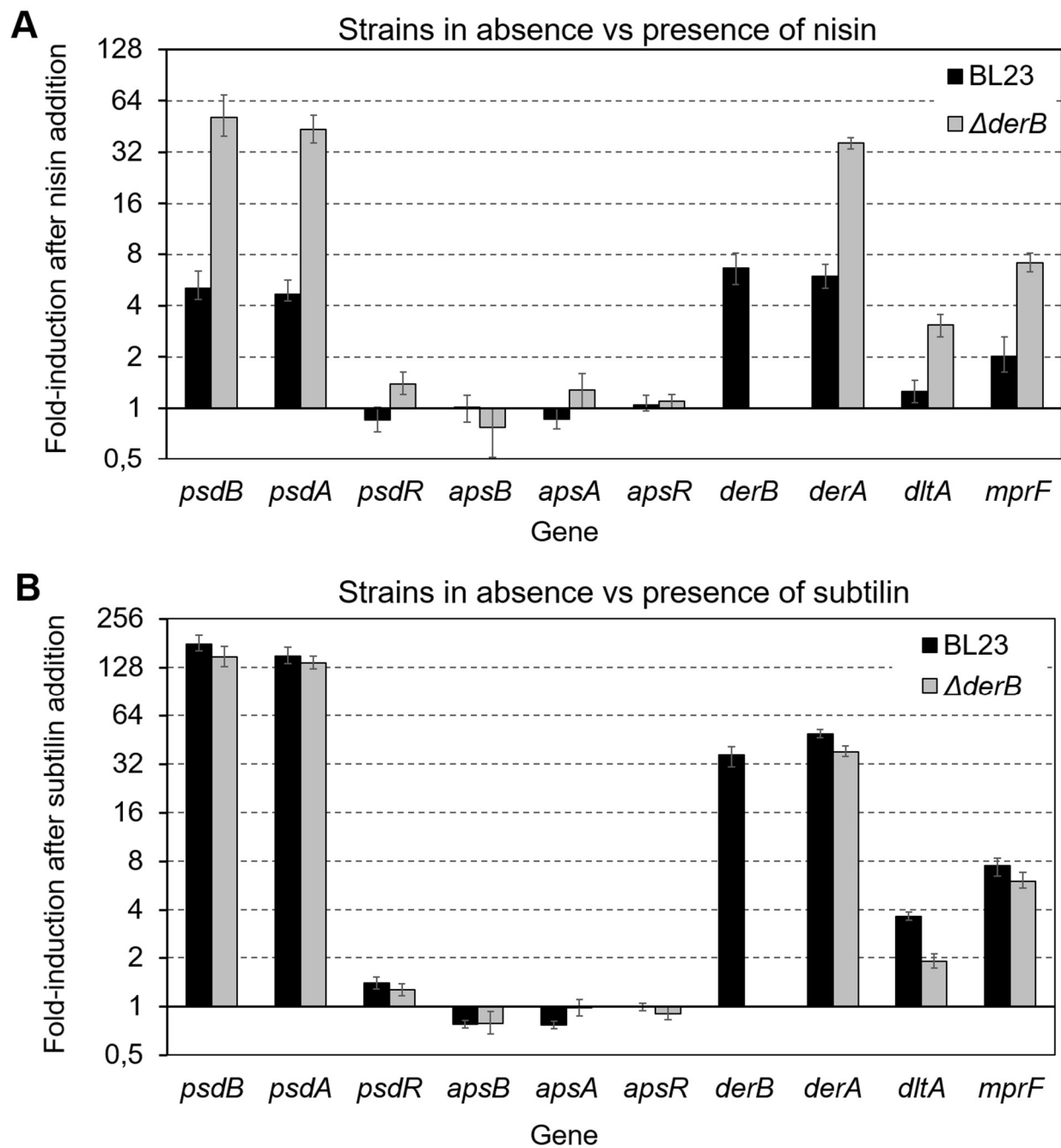


Figure 5: Expression of known AMP resistance determinants in *L. casei* BL23 and $\Delta derB$ mutant after nisin (A) and subtilin (B) addition. Transcript levels were determined by real-time RT-PCR 10 min after addition of 22.5 ng ml⁻¹ nisin (A) and 1% subtilin (B). X-fold induction was calculated relative to transcript levels in the same strain under reference conditions. Data are shown as mean \pm standard error of at least six replicates.

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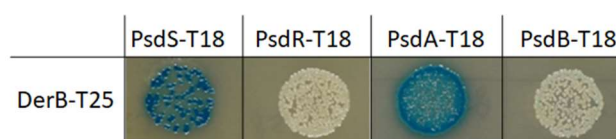


Figure 6: Bacterial two-hybrid analysis of the interactions of DerB with the PsdRSAB module. C-terminal and N-terminal translational fusions of the T18 and T25 domains of the adenylate cyclase CyaA of *B. pertussis* were created for each protein individually. Different hybrid fusions were tested in pairwise combinations in *E. coli* BTH101. The cells were spotted onto LB plates containing X-Gal (100 $\mu\text{g ml}^{-1}$), IPTG (1 mM), and antibiotics for selection. Pictures were taken after 48 h of incubation at 30 °C. The blue colonies indicate positive interaction results. For clarity, only a single representative pair is shown in the figure. See Figure S6 for the complete set of combinations.